Theses of the PhD dissertation entitled

The impact of dUTPase expression on DNA base composition and genome integrity

by

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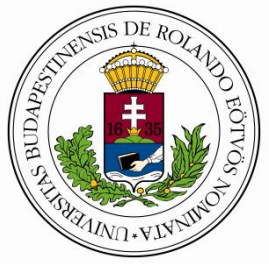

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1. Introduction

Cell cycle dependent and adequate nucleotide metabolism is responsible for maintaining the proper ratio of deoxynucleotide triphosphate (dNTP) pool components and meeting the dNTP requirements of DNA replication. In addition, exclusion of unusual bases from the DNA, carried out by preventive nucleotide hydrolases and DNA repair, is also of high importance. Abnormalities in the dNTP pool can result in DNA damage or DNA synthesis arrest that induces the so called DNA Damage Response (DDR). DDR can activate cell cycle block or apoptosis [1]. Thymidylate biosynthesis enables cells to utilize thymine bases instead of uracil for encoding information in DNA. A key process of thymidylate synthesis is the conversion of deoxyuridine-monophosphate (dUMP) to deoxythymidine-monophosphate (dTMP),carried out by thymidylate synthase (TS). dUTPase, also involved in thymidylate biosynthesis, hydrolyzes deoxyuridin-triphosphate to form dUMP, the substrate of TS. Moreover, dUTPase functions as a preventive nucleotide hydrolase to avoid uracil incorporation into DNA by eliminating dUTP pool. Uracil bases already present in DNA are removed by base excision repair enzymes including the most prominent UNG that is responsible for the majority of uracil excision [2]. UNG is common in most organisms except from fruit fly. My colleague, Dr. Angéla Békési showed previously that dUTPase expression is restricted to some specific tissues of *Drosophila* [3]. Due to absence of UNG and the spatio-temporal regulation of dUTPase expression, the fruit fly organism may provide a promising model to study uracil containing DNA in physiological conditions.

Since TS has a central role in thymidylate biosynthesis, it is a frequently used target of chemotherapy. TS inhibition results in a depleted deoxythymidine triphosphate (dTTP) concentration and mismatch repair mediated cytotoxicity [4]. Besides mismatch repair deficiency, toxicity can be decreased by dUTPase overexpression as well [5]. Meyers et al. hypothesized a model to explain the effects of 5-fluoropyrimidines frequently used in the clinics to inhibit TS [6]. According to the model, mismatches in DNA, apparent upon treatment, are consequences of the lack of dTTP, imbalanced dNTP pool and decreased replication fidelity. The model does not explain the role of dUTP in the resistance. dUTPase hydrolyze 5-fluoro-dUTP that is formed after 5-fluorouridine (5FdU) treatment thus excluding its incorporation into DNA, but the resulted 5-fluoro-dUMP is the direct inhibitor of TS. Therefore, the role of dUTPase in protection against TS inhibition is unclear.

DDR	- DNA damage response
TS	- thymidylate synthase
5FdU	- 5-fluoro-deoxyuridine
dU	- deoxyuridine
dT	- deoxythymidine

2. Experimental aims

In my theses, I intended to study the impact of dUTPase on the genome. Towards this aim, I decided to use the *Drosophila* model, since it lacks UNG and only expresses dUTPase in some tissues and stages. My other goal was to examine the mechanism by which dUTPase moderates the effects of TS inhibition. My relevant aims are listed below:

2.1. I wished to develop a method to quantify uracil content of DNA that can be routinely used and does not require complex instrumentation.

2.2. I wished to apply the method developed according to 2.1 on *Drosophila* samples to determine genomic uracil content and examine its dependence on dUTPase expression pattern.

2.3. I wished to examine the regulation mechanism responsible for dUTPase expression pattern at transcriptional level. Also, I intended to analyze the developmental role of this pattern. My colleague, Dr. Villő Muha showed that overall dUTPase silencing induced in larval stages leads to developmental arrest and lethality in pupal stage. To analyze significance of dUTPase expression pattern by other approach, I decided to analyze the effects of dUTPase overexpression in the organism.

2.4. I wished to examine the role of dUTPase in protecting DNA integrity. On one hand, my aim was to test whether the lethal effect of dUTPase silencing in Drosophila is related to the decrease of genome integrity.

On the other hand, I wanted to examine how dUTPase can decrease the toxicity of TS inhibition. In connection to this, I wanted to clarify whether exclusion of uracil or 5-fluorouracil from the genome or relieving dNTP imbalance can rescue the effects of TS inhibition.

3. Applied methods

To answer the question aimed in the **1st** point of the Experimental aims section, I utilized the DNA polymerase of the archeal *Pyrococcus furiosus* (Pfu) that was previously shown to be unable to elongate uracil-containing DNA templates [7]. The polymerase was applied in quantitative real-time PCR (qPCR). The method was verified on DNA isolated from wild type, UNG deficient or dUTPase and UNG double mutant *Escherichia coli*, and on DNA isolated from wild type or UNG knock out mouse embryonic fibroblasts (MEF) after 5FdU treatment.

To achieve the **2nd** aim, I applied the method above to quantify uracil accumulation in DNA isolated from wild type or dUTPase silenced *Drosophila* larval tissues or animals of different larval stages. I also analyzed the dUTPase expression pattern on several fruit fly tissues by immuncytochemistry to compare its relation to genomic uracil content.

To accomplish my **3rd** aim, I used the predicted dUTPase promoter to drive the expression of reporter genes having an easily detectable specific activity from cell lines or tissues of recombinant animals. In these systems I analyzed mutated and truncated versions of the dUTPase promoter as well. To disturb dUTPase expression pattern, we constructed transgenic *Drosophila* strains that can overexpress dUTPase by a tissue specific manner.

To address the **4th** aim in respect of the fruit fly, I induced dUTPase silencing in a wing imaginal disc specific manner and analyzed the adult wing phenotype. dUTPase silenced imaginal discs were stained by TUNEL assay to detect DNA fragmentation. DDR activity was addressed by immuncytochemistry against phospho-H2AX. Tissue proliferation was visualized by bromo-deoxyuridine assay.

To study the protecting effect of dUTPase against TS inhibition, I examined wild type or dUTPase silenced HeLa cell lines. The cells were treated with 5FdU or deoxyuridine (dU) at different concentrations and dose dependent cell viability was tested. Furthermore, the cells were treated with 5FdU at the determined IC_{50} concentration and complemented by dU or deoxythymidine (dT) to evaluate, how this components influence the cytotoxicity of TS inhibition.

4. Scientific results and conclusions

4.1. By applying Pfu DNA polymerase in qPCR, I could detect difference between DNA samples synthesized in the presence of different dUTP concentrations. I managed to show that the sensitivity of the method can be increased by specific primer pairs that designate longer amplicon size. Since any uracil residue in the template inhibits its replication, the longer the DNA segment of interest, the higher the chance to include uracil and inhibit polymerase. Detected uracil content of the synthesized templates was verified by an independent radioisotope-based method. The two method detected equivalent amount of uracil in the samples.

The method was also tested on physiological samples. Results showed that UNG deficiency has no detectable effect on uracil content of *E. coli* DNA, but a small change was observed if the culture was harvested at exponential growing phase. Deficiency in both dUTPase and UNG caused a dramatic effect in DNA composition by increasing uracil content to several thousand per million residues. In mammalian cells, lack of UNG did not affect genomic uracil content. UNG deficiency could only allow uracil accumulation in the case of TS inhibition carried out by 5FdU treatment. These results show agreement with previously published data [8–10]. Detailed description of this method is published in the *Nucleic Acids Research* journal (6.1/2).

4.2. Immuncytochemistry showed that dUTPase expression is only present in embryo, imaginal primordium tissues and central nervous system during larval stages and adult ovaries. These tissues are mostly undifferentiated proliferating tissues. Differentiated larval tissues do not proliferate, however, they perform endoreplication without cell division, and DNA synthesis occurs in absence of dUTPase. We showed that differentiated tissues, in contrast to undifferentiated tissues, accumulate uracil in the genome, at a level approximating thousand uracil basesper million residues. During larval stages, the uracil content of the DNA gets more and more increased probably due to the growing ratio of differentiated tissues in the animal. However, during pupal stage, the uracil content of the genome starts to decrease. During this stage, larval differentiated tissues are degraded by programmed cell death, and in parallel, undifferentiated tissues differentiate to form adult tissues. dUTPase silencing caused uracil accumulation in the genome of imaginal disc as well, at the same magnitude as observed in differentiated tissues. These results confirm that dUTPase expression primarily determines the uracil content of DNA. The relationship between dUTPase expression and

uracil-DNA appearance in the tissues is summarized on Figure 1. and published in PLoS Genetics (6.1/1).

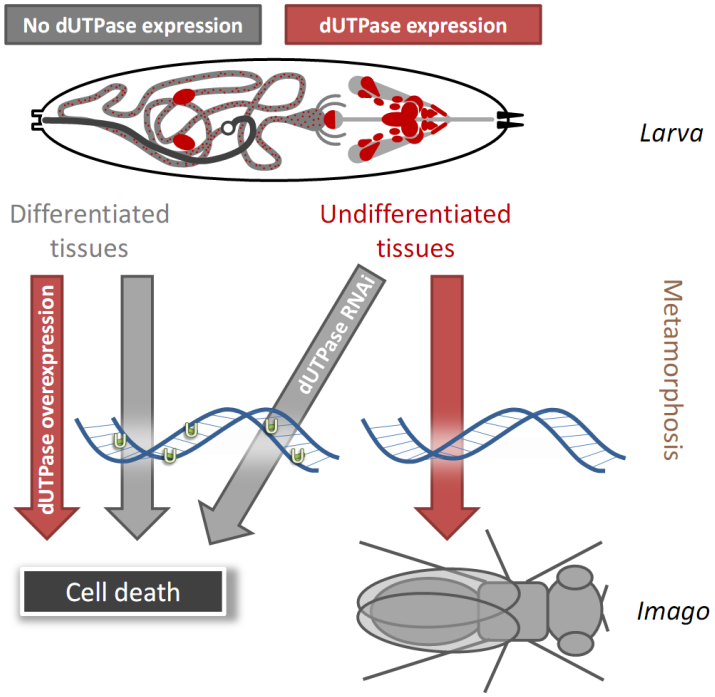

Figure 1. The consequences of dUTPase expression pattern in fruit fly

The figure summarizes our previous knowledge and the main results described in my current study. In larva, tissues marked as red show dUTPase expression. These are usually undifferentiated imaginal precursor tissues. Most of them, except from gonads, regulate dUTPase expression by the so called DNA replication-related element (DRE). According to our results, tissues that do not express dUTPase (e.g. repressing in differentiated tissues or silencing in imaginal discs) accumulate uracil in their genome. Besides this, dUTPase is essential for undifferentiated tissues to form adult tissues during metamorphosis. Absence of dUTPase results in the degradation of these tissues probably via DNA damage response (DDR). Differentiated tissues that downregulate dUTPase expression also degrade during metamorphosis, but this process does not depend on dUTPase.

4.3. Using promoter-reporter systems in S2 cells originating from *Drosophila* embryo, I was able to conclude that the dUTPase promoter includes two neighbouring regulating motif, the so-called DNA replication-related element (DRE). Transgenic Drosophila strains possessing promoter-reporter systems showed that the DRE containing fragment can induce gene expression in imaginal discs, central nervous system, and ventriculus ring gland of the larva. However, larval and adult gonads do not require DRE motifs to activate the promoter. DRE

regulates S-phase specific genes mostly related to replication. This is in agreement with the observation presented in section **4.2.**, namely dUTPase expression is restricted to proliferating tissues.

Since differentiated tissues that does not possess dUTPase expression, are degraded during pupal stage, and silencing of dUTPase affecting undifferentiated tissues results in pupal lethality, we assumed that dUTPase expression can determine the fate of some tissues during metamorphosis. However, dUTPase overexpression induced in differentiated tissues did not result in any visible phenotype compared to wild type. Thus, programmed cell death of differentiated tissues induced during metamorphosis may not necessarily require the absence of dUTPase. Cell fates depending on dUTPase expression are summarized on Figure 1.

4.4. The use of RNA interference allowed us to study the effect of dUTPase on genome integrity. Silencing of dUTPase in different segments of the wing imaginal disc resulted in degradation of the corresponding region of the adult wing. This observation implies that the effect of dUTPase silencing is cell autonomous. Besides this, we showed that silencing of dUTPase in wing discs resulted in DNA fragmentation supposedly induced by apoptosis, and DDR. Although DDR may induce cell cycle arrest; still, we could not detect any alterations between the proliferation patterns of silenced and wild type imaginal tissues. These results were summarized in the *PLoS Genetics* journal (6.1/1). Detailed description of the cell autonomous effect of dUTPase silencing is under revision at *Fly* journal (6.2/1).

The genome integrity protecting feature of dUTPase against TS inhibition was addressed on human cell lines. As my colleague, Gábor Merényi showed previously, dUTPase silencing decreased 5FdU sensitivity by several magnitude of concentration [5]. In contrast, dU did not affect cell viability even at millimolar concentration neither in the case of wild type nor the silenced cell line. Combined treatment with the nucleosides showed that while dT, as expected, decreased 5FdU toxicity, but dU surprisingly increased that. The sensitizing effect of dU against 5FdU did not depend on dUTPase expression. Nevertheless, we couldn't indicate any difference between dUTPase silenced and wild type cell lines regarding the effect of dU, results from combined nucleoside treatments suggests that the incorporation of dU into DNA is toxic as well, and may result in mismatches. According to this assumption, a specific role of dUTPase in resistance against TS inhibition is suggested to be restriction of the availability of imbalanced dNTP pool, especially dUTP accumulating due to TS inhibition, for DNA polymerase. Therefore, the model set up by Meyers et al. [6] can be completed as seen on Figure 2.

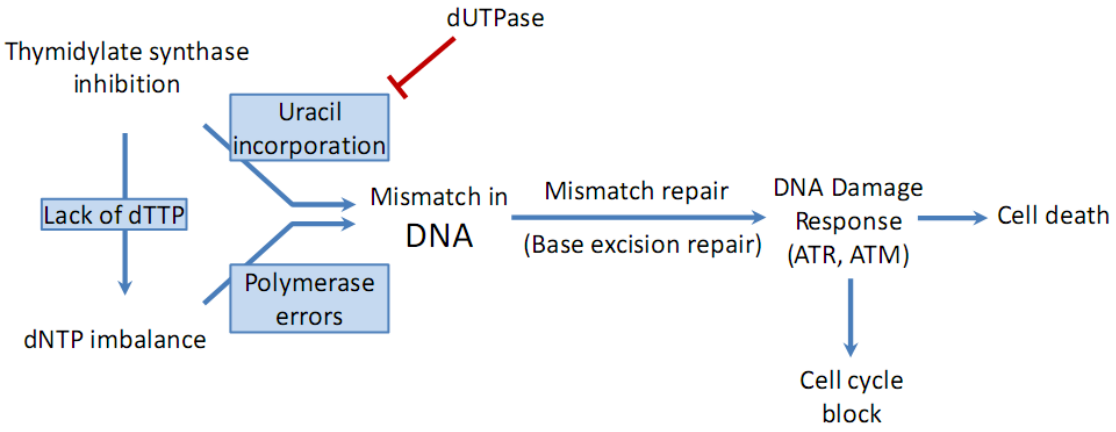

Figure 2. Supposed mechanism of thymidylate synthase inhibition induced genotoxicity in mammalian cells

The figure summarizes literature data and our experimental results gained from human cell lines. Frequent appearance of mismatches in DNA during thymidylate synthase inhibition is the consequence of two circumstances: imbalanced dNTP pool and high dUTP (or 5-fluoro-dUTP) concentration. The role of dUTPase is minimizing the latter one by decreasing the chance of DNA polymerase to find substrate. Thereby, in the presence of imbalanced dNTP pool, DNA polymerase can misincorporate fewer bases into DNA. Mismatches imply hazard for genome integrity mostly via mismatch repair. Severe repair induces the DNA damage response that can initiate cell cycle arrest or apoptosis. The scheme is the extension of the model hypothesized by Meyers et al [6].

5. References

1. Rai, P. Oxidation in the nucleotide pool, the DNA damage response and cellular senescence: Defective bricks build a defective house. *Mutation research* **703**, 71–81 (2010).
2. Vértessy, B. G. & Tóth, J. Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases. *Accounts of chemical research* **42**, 97–106 (2009).
3. Békési, A. *et al.* Developmental regulation of dUTPase in Drosophila melanogaster. *The Journal of biological chemistry* **279**, 22362–70 (2004).
4. Meyers, M. *et al.* DNA mismatch repair-dependent response to fluoropyrimidine-generated damage. *The Journal of biological chemistry* **280**, 5516–26 (2005).
5. Merényi, G. *et al.* Cellular response to efficient dUTPase RNAi silencing in stable HeLa cell lines perturbs expression levels of genes involved in thymidylate metabolism. *Nucleosides, nucleotides & nucleic acids* **30**, 369–90 (2011).
6. Meyers, M. *et al.* A role for DNA mismatch repair in sensing and responding to fluoropyrimidine damage. *Oncogene* **22**, 7376–88 (2003).

7. Fogg, M. J., Pearl, L. H. & Connolly, B. a Structural basis for uracil recognition by archaeal family B DNA polymerases. *Nature structural biology* **9**, 922–7 (2002).

8. Andersen, S. *et al.* Incorporation of dUMP into DNA is a major source of spontaneous DNA damage, while excision of uracil is not required for cytotoxicity of fluoropyrimidines in mouse embryonic fibroblasts. *Carcinogenesis* **26**, 547–55 (2005).

9. Lari, S.-U., Chen, C.-Y., Vertéssy, B. G., Morré, J. & Bennett, S. E. Quantitative determination of uracil residues in Escherichia coli DNA: Contribution of ung, dug, and dut genes to uracil avoidance. *DNA repair* **5**, 1407–20 (2006).

10. Luo, Y., Walla, M. & Wyatt, M. D. Uracil incorporation into genomic DNA does not predict toxicity caused by chemotherapeutic inhibition of thymidylate synthase. *DNA repair* **7**, 162–9 (2008).

6. List of publications

6.1. Accepted publications at peer-review journals (contributed equally)*

1. Villő Muha*, **András Horváth***, Angéla Békési, Mária Pukáncsik, Barbara Hodoscsek, Gábor Merényi, Gergely Róna, Júlia Batki, István Kiss, Ferenc Jankovics, Péter Vilmos, Miklós Erdélyi, Beáta G. Vértessy; *Uracil-containing DNA in Drosophila: Stability, Stage-specific Accumulation, and Developmental Involvement,* PLoS Genetics 2012, 8(6):e1002738

2. András Horváth, Beáta G. Vértessy; *A one-step method for quantitative determination of uracil in DNA by real-time PCR*, **Nucleic Acids Research** 2010, 38(21):e196

6.2. In press publications at peer-review journals

1. András Horváth, Angéla Békési, Villő Muha, Miklós Erdélyi, Beáta G. Vértessy; *Expending the Alphabet in the fruit fly: uracil enrichment in genomic DNA*, **Fly**, Accepted: December 12, 2012

6.3. Oral conference presentations (Name of presenter is underlined)

2012: <u>András Horváth</u>, Villő Muha, Angéla Békési, Júlia Batki, Péter Vilmos, István Kiss, Miklós Erdélyi, Beáta G. Vértessy

Cellular responses induced by uracil-DNA in Drosophila

IIIrd conference of Signal Transduction Division of Hungarian Biochemical Society, Esztergom, Hungary

2012: <u>András Horváth</u>, Villő Muha, Angéla Békési, Júlia Batki, Barbara Hodoscsek, Péter Vilmos, Ferenc Jankovics, István Kiss, Miklós Erdélyi, Beáta G. Vértessy

dUTPase is essential for genome stability and imaginal tissue development in Drosophila

FEBS 3+ Meeting, Opatija, Croatia

2011: Beáta G. Vértessy, Villő Muha, Angéla Békési, **András Horváth**, Gábor Merényi, Imre Zagyva, Judit Tóth, Ibolya Leveles

Uracil-DNA: repair and signal transduction

IXth Hungarian Congress on Genetics and XVIth Cell- and Developmental Biology Days, Siófok, Hungary

2011: Villő Muha, Angéla Békési, Róbert Gudor, **András Horváth**, Imre Zagyva, Beáta G. Vértessy.

Effect of dUTPase on Drosophila melanogaster development

IXth Hungarian Congress on Genetics and XVIth Cell- and Developmental Biology Days, Siófok, Hungary

2011: **András Horváth**, Villő Muha, Beáta G. Vértessy.

Deoxyuridine accumulation in oragnisms performing perturbed thymidylate metabolism

4th European Conference on Chemistry for Life Sciences (ECCLS), Budapest, Hungary

2010: **András Horváth**, Villő Muha, Angéla Békési, Judit Tóth, Judit Eszter Szabó, Ibolya Leveles, Anna Lopata, Miklós Erdélyi, Ferenc Jankovics, János Szabad, Beáta G. Vértessy

The World of uracil-DNA

Straub Days, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary

2008: István Tombácz, Tamás Schauer, Orbán Komornyi, **András Horváth**, Imre Boros

The role of RNA polymerase CTD phosphatase FCP1

Annual Meeting of Hungarian Biochemical Society, Szeged, Hungary

6.4. Poster conference presentations (Name of presenter is underlined)

2011: **András Horváth**, Villő Muha, Angéla Békési, Barbara Hodoscsek, István Kiss, Ferenc Jankovics, Miklós Erdélyi, Beáta G. Vértessy

Relation of factors influencing genomic uracil appearance and Drosophila development

3rd EMBO Meeting, Vienna, Austria

2010: **András Horváth**, Villő Muha, Beáta G. Vértessy

Uracil accumulation in DNA of organisms performing perturbed thymidylate metabolism

Straub Days, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary

2009: **András Horváth**, Beáta G. Vértessy

Detecting uracil in DNA by quantitative PCR

Annual Meeting of Hungarian Biochemical Society, Budapest, Hungary